Accelerated ripening of Caciocavallo Pugliese cheese with attenuated adjuncts of selected nonstarter lactobacilli

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ABSTRACT

The nonstarter lactic acid bacteria *Lactobacillus plantarum* CC3M8, *Lactobacillus paracasei* CC3M35, and *Lactobacillus casei* LC01, previously isolated from aged Caciocavallo Pugliese cheese or used in cheese-making, were used as adjunct cultures (AC) or attenuated (by sonication treatment) adjunct cultures (AAC) for the manufacture of Caciocavallo Pugliese cheese on an industrial scale. Preliminary studies on the kinetics of growth and acidification and activities of several enzymes of AAC were characterized in vitro. As shown by the fluorescence determination of live versus dead or damaged cells and other phenotype features, attenuation resulted in a portion of the cells being damaged and a portion of the cells being capable of growing with time. Compared with the control cheese (without adjunct cultures) and the cheese with AAC, the addition of AC resulted in a lower pH after manufacture, which altered the gross composition of the cheese. As shown by plate count and confirmed by random amplification of polymorphic DNA-PCR, the 3 species of nonstarter lactobacilli persisted during ripening but the number of cultivable cells varied between AC and AAC. Slight differences were found between cheeses regarding primary proteolysis. The major differences between cheeses were the accumulation of free amino acids and the activity levels of several enzymes, which were highest in the Caciocavallo Pugliese cheeses made with the addition of AAC. As shown by triangle test, the sensory properties of the cheese made with AAC at 45 d did not differ from those of the control Caciocavallo Pugliese cheese at 60 d of ripening. In contrast, the cheese made with AC at 45 d differed from both the Caciocavallo Pugliese cheese without adjuncts and the cheese made with AAC. Attenuated adjunct cultures are suitable for accelerating the ripening of Caciocavallo Pugliese cheese without modifying the main features of the traditional cheese.

Key words: adjunct culture, attenuation by sonication, Caciocavallo cheese

INTRODUCTION

Nonstarter lactic acid bacteria (NSLAB), mainly of the genus *Lactobacillus*, predominate during ripening of several varieties of cheeses (Crow et al., 2001). For some cheese varieties, NSLAB represent the main biological agents resulting in the typical sensory properties (De Angelis et al., 2001). Generally, the NSLAB remain uncontrolled in industrial cheese making because off-flavors, formation of biogenic amines, and blowing may occur if the dominant strains are altered (Crow et al., 2001). Although the control of the NSLAB biota could be pursued through the use of adjunct cultures, this procedure seems to be outdated due to several risks such as excessive acidification during manufacture and the variable effects on texture and sensory properties of the cheeses.

Over the last decade (Madkor et al., 2000; Upadhyay et al., 2007), adjuncts of mesophilic nonstarter lactobacilli have also been used as attenuated cultures. Several methods have been used to achieve attenuation, including heat treatment, freezing and thawing, freeze- or spray-drying, lysozyme treatment, use of solvents, and high-pressure treatment (Klein and Lortal, 1999; Upadhyay et al., 2007). Recently, sonication has been proposed for the attenuation of adjuncts of mesophilic nonstarter lactobacilli used in making Italian Caciotta-type cheese (Di Cagno et al., 2011). Sonication appeared to be a novel and efficient method to attenuate adjunct cultures, because it was simple, rapid, and reproducible and did not alter the activity of intracellular enzymes (Di Cagno et al., 2011). Overall, attenuated cultures of mesophilic nonstarter lactobacilli should not have the capacity to transport, phosphorylate, and hydrolyze lactose during cheese manufacture, but they must contain enzymes that influence cheese quality during ripening. Only a few studies (Upadhyay et al., 2007) have demonstrated, in part, how attenuation interferes with cell viability and how attenuated cultures may recover capacities of growth and acidification in vitro

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and during cheesemaking. The ultimate goal of using selected and attenuated strains of NSLAB is to accelerate and improve flavor and texture development during cheese ripening.

None of the previous studies has considered the use of attenuated adjuncts of selected nonstarter lactobacilli during the manufacture of semi-hard *pasta filata* cheeses. Stretching of the curd in hot water is undoubtedly a selective technology parameter, which may influence the performance of attenuated adjunct cultures. Caciocavallo, a typical *pasta filata* cheese, is manufactured in 3 different regions: the Balkans, Russia, and Italy. Different varieties of Caciocavallo cheese are manufactured in southern Italy, including Caciocavallo Silano, Molisano, Pugliese, and Corleonese. Caciocavallo Pugliese has very wide market popularity—approximately 9,234 t are manufactured per year (www.istat.it). The cheese is made from raw cow milk, using natural whey or freeze-dried primary starters, and a temperature $>70^{\circ}\text{C}$ is used during plasticization of the curd, which results in the formation of paracasein fibers of relatively high tensile strength that impart stringiness to the cheese (Gobbetti et al., 2002). Caciocavallo Pugliese cheese may be sold after just 2 wk of ripening or, more commonly, after 2 to 4 mo. The cheese typically has an oval or flask-like shape due to the way the curd is handled during stretching. After manufacture, the end of the body curd is tied with a cord, which is attached to a long, robust stick throughout the ripening period. The ripened cheese weighs 1 to 2.5 kg, the rind is pale yellow, and the interior is compact with small eyes. Defects of texture and flavor may sometimes appear due to uncontrolled fermentation or enzyme activities and because of the use of raw cow milk.

This study describes the *in vitro* kinetics of growth and acidification of attenuated (by sonication treatment) adjuncts of 3 selected nonstarter lactobacilli used to accelerate the ripening of Caciocavallo Pugliese cheese. Traditional cheesemaking and the use of nonattenuated selected lactobacilli strains were compared with respect to compositional, microbiological, biochemical, and sensory characteristics.

MATERIALS AND METHODS

Mesophilic Nonstarter Lactobacilli

Lactobacillus plantarum CC3M8 and *Lactobacillus paracasei* CC3M35, which were previously isolated from aged Caciocavallo cheeses, and *Lactobacillus casei* LC01, belonging to the Culture Collection of the Department of Biologia e Chimica Agro-Forestale ed Ambientale (Bari University, Bari, Italy) and previously used in

cheese making (Di Cagno et al., 2011), were identified by partial sequencing of the 16S rRNA. The 3 strains were selected based on activities of aminopeptidase type N, proline iminopeptidase, endopeptidase type O, glutamate dehydrogenase, and cystathionine lyase (Di Cagno et al., 2011) and used as adjunct attenuated cultures.

Adjunct and Attenuated Adjunct Cultures

After overnight cultivation in de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Basingstoke, UK), selected mesophilic nonstarter lactobacilli used as adjunct cultures (AC) were harvested by centrifugation ($10,000 \times g$, 10 min at 4°C), washed twice with sterile 50 mM potassium phosphate buffer, pH 7.0, and resuspended in sterile distilled water at the cell count of $\sim 9.0 \log_{10}$ cfu/mL. This cell suspension was used to inoculate sterile skim milk (Oxoid). To obtain attenuated adjunct cultures (AAC), the same volume of harvested cells from MRS broth was resuspended in distilled water to the same cell count ($\sim 9.0 \pm 0.2 \log_{10}$ cfu/mL), and the cell suspension was subjected to sonication in an ice bath. Sonication was carried out by using a Vibra-Cell sonicator (Sonic and Materials Inc., Danbury, CT) with a microtip setting (sonic power 375 W; output control 5) for 45 min (3 cycles, 15 min/cycle, 15-min interval between cycles). As estimated by plate count, sonication decreased the cell number to $\sim 5.1 \pm 0.1 \log_{10}$ cfu/mL. Then, AC (final cell count of $\sim 7.0 \pm 0.3 \log_{10}$ cfu/mL) and AAC (cell count, before sonication treatment, of $\sim 9.0 \pm 0.2 \log_{10}$ cfu/mL, which corresponded to $\sim 5.1 \pm 0.1 \log_{10}$ cfu/mL of cultivable cells) were individually incubated in sterile skim milk (30°C for 80 h) or MRS broth (30°C for 24 h). Kinetics of growth and acidification were determined. Growth was monitored by plating on MRS agar at 30°C for 48 h. Acidification was directly determined by a Foodtrode electrode (Hamilton, Bonaduz, Switzerland). Growth and acidification data were modeled according to the Gompertz equation, as modified by Zwietering et al. (1990):

$$y = k + A \exp \{ -\exp [(\mu_{\max} \text{ or } V_{\max} e/A)(\lambda - t) + 1] \},$$

where y is growth expressed as $\log_{10}$ cfu/mL per hour or acidification extent expressed as dpH/dt (units of pH/h) at the time t ; k is the initial level of the dependent variable to be modeled ($\log_{10}$ cfu/mL or pH units); A is the difference in cell count or pH (units) between inoculation and the stationary phase; $\mu_{\max}$ or $V_{\max}$ is the maximum growth rate expressed as $\Delta \log_{10}$ cfu/mL per hour or the maximum acidification rate

expressed as dpH/h , respectively; λ is the length of the latency phase expressed in hours; and t is the time. The experimental data were modeled through the nonlinear regression procedure of the statistics package Statistica for Windows (Statsoft, Tulsa, OK).

Determination of Cell Membrane Integrity

Cell membrane integrity of AAC was estimated using the LIVE/DEAD *BacLight* Bacterial Viability Kit (Molecular Probes Inc., Cambridge Bioscience, Cambridge, UK), according to the manufacturer's instruction. The analysis determines the structural integrity of the bacterial membrane using the green-fluorescing dye SYTO 9 and the red-fluorescing propidium iodide; SYTO 9 labels all cells, whereas propidium iodide penetrates only cells with a damaged membrane, causing a decrease in SYTO 9 fluorescence when both dyes are present. Stained bacterial suspensions were observed using a Leica LDMC (Leica Microsystems SpA, Milano, Italy) with a $60\times$ objective. Images were analyzed and cell numbers were quantified from the images using Image-Pro Plus image analysis software (Media Cybernetics Inc., Silver Spring, MD; Biggerstaff et al., 2006).

Enzyme Activities

Enzyme activities were determined during incubation of AC and AAC in sterile skim milk and during ripening of Caciocavallo Pugliese cheese. In both cases, enzyme activities were determined in the water-soluble extracts of milk or cheese prepared according to the method of Kuchroo and Fox (1982), with further modifications (Di Cagno et al., 2006). Aminopeptidase (EC 3.4.11.11) type N activity on Leu-*p*-nitroanilide (*p*-NA), proline iminopeptidase (EC 3.4.11.9) activity on Pro-*p*-NA, and endopeptidase type O (EC 3.4.23) activity on Z-Gly-Pro-NH-trifluoromethyl-coumarin were determined as described by Gobbetti et al. (1999). An arbitrary unit of enzymatic activity was defined as the amount of enzyme that caused an increase in absorbance at 410 nm per minute of 1 (aminopeptidase type N), 0.1 (proline iminopeptidase), and 0.1 (endopeptidase type O) at 37°C and pH 7.0. Glutamate dehydrogenase (EC 1.4.1.2) activity was determined on glutamate by measuring the glutamate-dependent reduction of NADP or NAD at 340 nm, as described by De Angelis et al. (2010). An arbitrary unit of enzymatic activity was defined as the amount of enzyme that gave an increase of absorbance of 0.1 per minute at 37°C and pH 7.0. Cystathionine lyase (EC 4.4.1.1) activity was determined by measuring the amount of ketoacids, ammonia, and free thiols released from cystathionine, as described by De Angelis et al. (2002). An arbitrary

unit of enzymatic activity was defined as the amount of enzyme that caused an increase of absorbance (412 nm) of 1 per minute at 37°C and pH 7.0.

Manufacture of Caciocavallo Pugliese Cheese

The cow milk used in cheesemaking had the following characteristics: lactose 4.9%, protein 3.3%, fat 3.6%, and pH 6.6. Daily, the same milk was used for the manufacture of the 3 types (control cheese without addition, cheese with adjunct cultures of AC or AAC) of cheese at an industrial plant (Ignalat, Noci Bari, Italy; vat of ~ 200 L). Cheesemaking was carried out on 3 consecutive days (total of 3 batches for each type of cheese) using cheese milk from daily milking. When used for cheesemaking (AC or AAC), the 3 mesophilic nonstarter lactobacilli were added as a mixture. Raw cow milk was heated at 37°C and inoculated with (1) freeze-dried (Sacco, Cadorago, Como, Italy) primary starter *Streptococcus thermophilus* (initial cell count of $\sim 7.0 \pm 0.2 \log_{10}$ cfu/mL of milk; control cheese, CC), (2) primary starter and AC (initial cell count of each species of $\sim 7.2 \pm 0.2 \log_{10}$ cfu/mL of milk), or (3) primary starter and AAC (cell count of each species, before sonication treatment, of $\sim 9.1 \pm 0.2 \log_{10}$ cfu/mL, which corresponded to $\sim 5.2 \pm 0.1 \log_{10}$ cfu/mL of cultivable cells in the milk). After 30 min at 37°C , liquid calf rennet (35 mL/100 L) was added and coagulation took place within 30 min. The coagulum was first cut coarsely by hand, held under whey at 37°C for 2 h, and then reduced mechanically to particles of 1.5 to 2 cm. When the curd reached a pH of 5.3 (~ 1 h), it was stretched in hot water (80°C). Cheeses were salted in brine (28%, wt/vol, NaCl) for 8 h and ripened at 12°C for 2 mo. The weight of the cheeses was approximately 1 kg.

Microbiological Analyses

Samples (20 g) of Caciocavallo cheese were diluted in 180 mL of sodium citrate (2%, wt/vol) solution and homogenized with a Stomacher Lab-Blender 400 (PBI International, Milan, Italy). Serial dilutions were made in quarter-strength Ringer's solution and plated on M17 or MRS agar (Oxoid) to enumerate presumptive thermophilic streptococci (42°C for 48 h) or mesophilic lactic acid bacteria (30°C for 48–72 h, under anaerobiosis). Colonies were collected from the plates (MRS agar incubated at 30°C for 48–72 h, under anaerobiosis) of the highest dilutions of each cheese to detect the mesophilic nonstarter lactobacilli used as adjuncts. Gram-positive, catalase-negative, nonmotile rod isolates were cultivated in MRS broth (Oxoid) at 30°C for 24 h and restreaked into MRS agar.

Random Amplified Polymorphic DNA-PCR Analysis

Genomic DNA was extracted as described by de Los Reyes-Gavilán et al. (1992) from 2 mL of overnight culture grown in MRS at 30°C. Two primers (Invitrogen Life Technologies, Milan, Italy), with arbitrarily chosen sequences (P4, 5'-CCGCAGCGTT-3' and M13, 5'-GAGGGTGGCGGTTCT-3'; De Angelis et al., 2001; Rossetti and Giraffa, 2005) were used singly in 2 series of amplification. The reaction mixture contained 200 $\mu\text{mol/L}$ of each dNTP, 1 to 2 $\mu\text{mol/L}$ of primer, 1.5 to 3 $\mu\text{mol/L}$ of MgCl_2 , 1.25 U *Taq* DNA polymerase (Invitrogen), 2.5 μL of PCR buffer, 25 ng of DNA, and sterile double distilled water to 25 μL . For amplifications with primer P4, the PCR program comprised 45 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 35°C, and elongation for 2 min at 72°C; the cycles were preceded by denaturation at 94°C for 4 min and followed by elongation at 72°C for 5 min. For primer M13, amplification reactions were carried out according to the protocol described by Giraffa et al. (2000): one cycle at 94°C for 60 s (denaturing), 42°C for 20 s (annealing), and 72°C for 2 min (elongation). The PCR products were separated by electrophoresis (2 h at 130 V) on 1.5% (wt/vol) agarose gel (Invitrogen), and the DNA was detected by UV transillumination after staining with ethidium bromide (0.5 $\mu\text{g/mL}$). The molecular weight of the amplified DNA fragments was estimated by comparison with the 1 kb Plus DNA Ladder (Invitrogen) ranging from 100 to 12,000 bp.

Random amplification of polymorphic DNA (RAPD)-PCR analyses were carried out for typing isolates during ripening of Caciocavallo Pugliese cheese to detect AAC. The reproducibility of RAPD fingerprints was assessed by comparing the PCR products obtained from 3 separate cultures of the same strain. After typing, isolates were identified by 16S rRNA gene sequence analysis (De Angelis et al., 2006).

Compositional and Biochemical Analyses

Samples of cheese were analyzed for protein (macro-Kjeldahl; IDF, 1964), fat (Gerber method; IIRS, 1955), moisture (oven drying at 102°C; IDF, 1982), and salt (Fox, 1963).

The pH 4.6-insoluble and pH 4.6-soluble nitrogen fractions of the cheeses were analyzed by urea-PAGE and reverse phase-HPLC as described by Di Cagno et al. (2003) and Gobetti et al. (2002), respectively. Total and individual free amino acids (FAA) from the water-soluble extracts of cheeses were determined by the Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, UK; Di Cagno et al., 2007).

Sensory Analysis

Sensory analysis was carried out on Caciocavallo Pugliese cheeses made without addition (CC) after 60 d of ripening and with AAC or AC after 45 d of ripening. The triangle test was applied (ISO, 2004), choosing $\alpha = 0.05$, $\beta = 0.05$, $P_d = 40\%$ (where α is the probability of concluding that a perceptible difference exists when one does not, β is the probability of concluding that no perceptible difference exists when one does, and P_d is the proportion of assessments in which a perceptible difference is detected between the 2 products), and 42 assessors to attempt the sensory evaluation. Assessors were chosen from students, researchers, and employees of the Department of Biologia e Chimica Agro-Forestale ed Ambientale (Bari, Italy), with an equal distribution of men and women and ranging in age between 21 and 45 yr. Six different combinations for each triangle test (CC-CC-AAC, CC-AAC-CC, AAC-CC-CC, AAC-AAC-CC, AAC-CC-AAC, CC-AAC-AAC; CC-CC-AC, CC-AC-CC, AC-CC-CC, AC-AC-CC, AC-CC-AC, CC-AC-AC; and AC-AC-AAC, AC-AAC-AC, AAC-AC-AC, AAC-AAC-AC, AAC-AC-AAC, AC-AAC-AAC) were considered and distributed in randomized order to the assessors. The forced-choice procedure was applied. For the sessions, cheese samples were randomly coded and served, in aliquots of 20 g together with still water, to assessors placed in separate rooms for unbiased evaluation.

Statistical Analysis

All cheese analyses were carried out twice for each of the 3 batches (total 6 analyses for each type of cheese). Data were subjected to one-way ANOVA (SAS Institute, 1985); pair-comparison of treatment means was achieved by Tukey's procedure at $P < 0.05$, using the statistical software Statistica for Windows (Statistica 6.0, 1998). For sensory analysis, the statistical tables annexed to ISO (2004) and ISO (2008) were used.

RESULTS AND DISCUSSION

In Vitro Kinetics of Growth and Acidification

Lactobacillus casei LC01, *Lactobacillus plantarum* CC3M8, and *Lactobacillus paracasei* CC3M35 were previously isolated from aged Caciocavallo Pugliese cheese or used in cheesemaking (Di Cagno et al., 2011) and were selected based on various enzyme activities (see Materials and Methods).

Strains were singly subjected to attenuation by sonication treatment, and the kinetics of growth and acidification

fication were assayed on sterile skim milk (30°C for 80 h) and MRS broth (30°C for 24 h). Untreated AC grew into sterile milk from $\sim 7.0 \log_{10}$ cfu/mL to an average value of $9.5 \pm 0.2 \log_{10}$ cfu/mL. Strains differed in the latency phase of growth, which may reflect the different capacity to adapt to milk during the early phase of cheese manufacture. The lengths of the latency phase (λ) were 0.51 ± 0.08 , 1.45 ± 0.11 , and 3.88 ± 0.09 h for *L. casei* LC01, *L. plantarum* CC3M8, and *L. paracasei* CC3M35, respectively. The range for $\mu_{\max}$ was 0.11 to $0.16 \Delta \log_{10}$ cfu/mL per hour. Attenuated adjunct cultures started from an initial cell count of $\sim 5.1 \pm 0.1 \log_{10}$ cfu/mL and, after 80 h of incubation on sterile skim milk, reached an average value of cell viability ($9.1 \pm 0.3 \log_{10}$ cfu/mL) only slightly lower than that of untreated cells. Compared with that in AC cells, the values of λ in AAC increased markedly to 3.17 ± 0.15 , 2.69 ± 0.1 , and 5.39 ± 0.09 h for *L. casei* LC01, *L. plantarum* CC3M8, and *L. paracasei* CC3M35, respectively. The increase in the latency phase was related to sonication treatment and, likely, to the injury of cells. Under these conditions, the growth of *L. plantarum* CC3M8 was the fastest. The range for $\mu_{\max}$ was 0.14 to $0.18 \Delta \log_{10}$ cfu/mL per hour. By the end of the incubation period, the values of Δ pH were almost similar for the untreated AC (range of 1.85 to 2.08 units) and sonicated AAC (range of 1.92 to 2.32 units) cells (Figure 1). Nevertheless, the kinetics of acidification were very different. Moreover, because of the sonication treatment, the latency phase of acidification (λ) increased markedly (4 to 20 times) from AC to AAC: 3.99 ± 0.11 to 19.32 ± 1.05 , 4.61 ± 0.12 to 45.34 ± 1.72 , and 2.36 ± 0.05 to 39.78 ± 1.44 h for *L. casei* LC01, *L. plantarum* CC3M8, and *L. paracasei* CC3M35, respectively. The values of $V_{\max}$ varied only slightly between AC (range of 0.08 to 0.11 dpH/h) and AAC (range of 0.04 to 0.11 dpH/h). The same trend was found during growth on MRS broth (data not shown).

We compared AC and AAC (same cell count of $\sim 9.0 \log_{10}$ cfu/mL before sonication treatment) in vitro for activities of several enzymes related to cheese ripening (Gobbetti et al., 1999; Table 1). Except for activity of glutamate dehydrogenase of *L. casei* LC01 and *L. plantarum* CC3M8, and that of endopeptidase type O of *L. plantarum* CC3M8, all other activities (especially aminopeptidase type N, proline iminopeptidase, and cystathionine lyase) were markedly higher in AAC compared with AC. It appeared that treatment by sonication severely damaged the cell membrane, which resulted in the release of intracellular enzymes and thus higher activities compared with AC.

As shown by plate count, the number of cultivable cells of *L. plantarum* CC3M8 increased progressively from $5.1 \pm 0.1 \log_{10}$ cfu/mL (at 0 h) to $8.8 \pm 0.2 \log_{10}$

Figure 1. Kinetics of acidification (Δ pH) of *Lactobacillus casei* LC01 (A), *Lactobacillus plantarum* CC3M8 (B), and *Lactobacillus paracasei* CC3M35 (C) incubated as adjunct (O; cell count of $\sim 7.0 \log_{10}$ cfu/mL) or attenuated adjunct cultures (Δ ; cell count before sonication treatment of $\sim 9.0 \pm 0.2 \log_{10}$ cfu/mL, corresponding to $\sim 5.1 \pm 0.1 \log_{10}$ cfu/mL of cultivable cells) in sterile skim milk at 30°C for 80 h. Data are the mean of 3 independent experiments ($n = 3$) analyzed in duplicate.

Table 1. Enzyme activities¹ of *Lactobacillus casei* LC01, *Lactobacillus plantarum* CC3M8, and *Lactobacillus paracasei* CC3M35 used as adjunct cultures or attenuated adjunct cultures

Enzyme	Adjunct culture			Attenuated adjunct culture		
	LC01	CC3M8	CC3M35	LC01	CC3M8	CC3M35
Aminopeptidase type N	17 ± 0.68 ^a	6.2 ± 0.25 ^b	160 ± 6.4 ^a	138 ± 5.52 ^a	11.5 ± 0.46 ^b	440 ± 17.6 ^a
Proline iminopeptidase	0.07 ± 0.003 ^c	0.55 ± 0.02 ^c	0.57 ± 0.02 ^b	0.98 ± 0.40 ^d	0.72 ± 0.03 ^c	0.8 ± 0.03 ^c
Endopeptidase type O	0.02 ± 0.001 ^c	0.03 ± 0.001 ^c	0.005 ± 0.001 ^c	0.24 ± 0.40 ^d	0.03 ± 0.001 ^d	0.03 ± 0.001 ^d
Glutamate dehydrogenase	4 ± 0.16 ^b	0 ± 0.00 ^d	0 ± 0.00 ^d	3.3 ± 0.13 ^c	0 ± 0.00 ^e	2.25 ± 0.10 ^b
Cystathionine lyase	0 ± 0.00 ^c	12.1 ± 0.49 ^a	0.1 ± 0.0 ^b	17.2 ± 0.70 ^b	34.3 ± 1.37 ^a	0.44 ± 0.02 ^c

^{a-e}Means within a row with different superscript letters are significantly different ($P < 0.05$).

¹Arbitrary units (see Materials and Methods). Values represent the average of 3 triplicates (±SD) from 2 repeated cell cultivations.

cfu/mL (at 24 h) during incubation in MRS broth. The analysis by bacterial viability kit and the related quantification with the Image-Pro Plus image software (Figure 2) showed that the number of intact cells (green fluorescing) progressively increased to $\sim 8.5 \pm 0.1 \log_{10}$ cfu/mL (24 h). After inoculation, the estimated number of dead or damaged cells (red fluorescing) of *L. plantarum* CC3M8 was $\sim 7.0 \log_{10}$ cfu/mL, which did not vary throughout 24 h of incubation. These findings showed that a considerable proportion of *L. plantarum* CC3M8 cells were subjected to cell membrane injury. Similar results were found for the other 2 strains of lactobacilli (data not shown). Only a few studies (Upadhyay et al., 2007) have characterized in vitro the effect of attenuation treatment and determined the further behavior of attenuated cells. The lactase dehydrogenase was previously used as the marker to show cell lysis. In this study, the use of the LIVE/DEAD BacLight Bacterial Viability Kit, together with analysis of enzyme activities, allowed us to estimate the proportions of intact and damaged cells of AAC, which may differently affect the cheese during ripening.

Cheese Compositional and Microbiological Analyses

As expected, the use of AC affected the pH in the manufacture of Caciocavallo Pugliese cheese (Table 2). Compared with the CC (pH 5.23 ± 0.10) and AAC (pH 5.25 ± 0.12) cheeses, the cheese with AC added had a pH of 5.03 ± 0.08 at the beginning of ripening. Overall, these differences of pH were almost constant until 45 d of ripening. During the last 20 d of ripening, the values of pH slightly increased for all cheeses and the differences among them were attenuated, from 5.19 ± 0.07 and 5.14 ± 0.09 (CC and AAC) to 5.05 ± 0.03 (AC). The use of AAC did not affect the pH or gross composition of the cheese compared with CC, and this is one of the main prerequisites for AAC to be successfully used (Madkor et al., 2000; Upadhyay and McSweeney, 2003). Impaired AAC could be due to the effects of sonication treatment on enzymes involved in

lactose phosphorylation or transportation (Casal and Gomez, 1999). At the end of ripening (60 d), the values of moisture, fat, protein, and salt for CC and AAC cheeses were in the range of those usually found during Caciocavallo Pugliese cheesemaking (Ottogalli, 2001). The cheese made with AC showed a lower value of moisture ($33.8 \pm 1.6\%$), which slightly interfered with the concentrations of fat and protein.

Caciocavallo Pugliese cheese was made with raw cow milk, and stretching of the curd in hot water (80°C) was performed. The latter step had a negative effect on the added mesophilic lactic acid bacteria (Table 3). The CC cheese had the lowest initial cell count ($3.5 \pm 0.3 \log_{10}$ cfu/g) of presumptive nonstarter mesophilic lactic acid bacteria, which increased during ripening to a value of $7.6 \pm 0.3 \log_{10}$ cfu/g. The cheese manufactured with the addition of AC had the highest ($P < 0.05$) initial number of presumptive nonstarter mesophilic lactic acid bacteria ($6.2 \pm 0.3 \log_{10}$ cfu/g), which increased to reach a value of $8.7 \pm 0.3 \log_{10}$ cfu/g at the end of ripening. At the beginning of ripening, the cheese manufactured with the addition of AAC contained $4.1 \pm 0.3 \log_{10}$ cfu/g of cultivable nonstarter lactic acid bacteria. After whey drainage, a considerable proportion of AAC presumably became entrapped in the cheese curd drainage (Madkor et al., 2000; Upadhyay and McSweeney, 2003). The cell numbers of AAC increased progressively, especially between 15 and 30 d, and remained almost constant thereafter at $8.4 \pm 0.3 \log_{10}$ cfu/g. Presumptive mesophilic nonstarter lactic acid bacteria randomly isolated from the highest MRS agar plate dilution were subjected to RAPD-PCR typing. Cheeses manufactured with the addition of AC or AAC contained *L. casei* LC01, *L. plantarum* CC3M8, and *L. paracasei* CC3M35, which were capable of withstanding stretching of the curd. Previously (Morea et al., 2007), strains of *Lactobacillus parabuchneri* used as adjunct cultures were not recovered during ripening of Caciocavallo Pugliese cheese. As shown for other attenuation treatments (Upadhyay et al., 2007), a sufficient number of intact cells was probably capable of further growth

Figure 2. Fluorescing *Lactobacillus plantarum* CC3M8 cells. Intact and dead/damaged cells are stained green (row 1) and red (row 2), respectively. *Lactobacillus plantarum* CC3M8 was grown in de Man, Rogosa, and Sharpe (MRS) broth at 30°C for 0 (A and B), 7 (C and D), 10 (E and F), and 24 (G and H) h.

during cheese ripening. The opposite was found following attenuation by freeze-shocking, heat-shocking, or spray-drying on *Lactobacillus helveticus* and *L. casei* strains (Madkor et al., 2000). Overall, cheeses made with additions of AC or AAC contained a similar number of NSLAB and the starter species, which are usually found during ripening of Caciocavallo Pugliese or other varieties of Caciocavallo cheeses (Gobbetti et al., 2002; Piraino et al., 2005). The number of presumptive thermophilic streptococci was almost the same for the 3 types of Caciocavallo Pugliese cheese (Table 3). The cell count of thermophilic streptococci decreased slightly during ripening and reached values of 8.7 ± 0.3 to $8.9 \pm 0.2 \log_{10}$ cfu/g at 60 d, similar to values were found in other Caciocavallo cheeses manufactured in southern Italy (Piraino et al., 2005). In contrast to previous studies (Di Cagno et al., 2011), NSLAB, in either AC or AAC, did not interfere with the viability of primary starters (in this case, *Streptococcus thermophilus*).

Proteolysis

Urea-PAGE electrophoresis of the pH 4.6-insoluble nitrogen fraction showed a few differences between pri-

mary proteolysis of the 3 cheeses (Figure 3A). Although some α_{S1} -CN persisted in the CC, it was completely degraded after 60 d in cheeses ripened with the additions of AC or AAC. At the end of ripening, a large amount of nonhydrolyzed β -CN persisted in all cheeses. As shown in previous studies (Farkye and Fox, 1990; Milanovic et al., 1998; Gobbetti et al., 2002), plasmin activity would be expected during primary proteolysis of ripened *pasta filata* cheeses. Although a debatable point, chymosin should be fully inactivated by scalding at 80°C. Starting from 30 d, the urea-PAGE electrophoresis of the pH 4.6-soluble nitrogen fraction slightly differed between CC and the cheeses manufactured with AAC (Figure 3B). In particular, the cheese made with AAC showed less intense bands of polypeptides. In contrast to other studies (Madkor et al., 2000), a moderate direct or indirect effect of AC and AAC was found during primary proteolysis. The same results were found by RP-HPLC analysis (data not shown).

At the beginning of ripening, no significant ($P > 0.05$) differences were found for the total concentration of FAA among the 3 cheeses (634.9 ± 25 to 685.7 ± 39 mg/kg; Table 4). At the end of ripening, the highest increase ($10,060.1 \pm 40$ mg/kg) was found for the cheese

Table 2. Main chemical composition¹ during ripening of Caciocavallo Pugliese cheeses

Item	Control cheese					Cheese with adjunct culture					Cheese with attenuated adjunct culture				
	0 d	15 d	30 d	45 d	60 d	0 d	15 d	30 d	45 d	60 d	0 d	15 d	30 d	45 d	60 d
pH	5.23 ± 0.1 ^a	5.08 ± 0.15 ^b	4.9 ± 0.11 ^c	4.82 ± 0.09 ^d	5.19 ± 0.07 ^b	5.03 ± 0.08 ^{bc}	4.87 ± 0.07 ^d	4.72 ± 0.1 ^e	4.6 ± 0.13 ^f	5.05 ± 0.03 ^{bc}	5.25 ± 0.12 ^a	5.1 ± 0.13 ^b	4.92 ± 0.07 ^c	4.8 ± 0.03 ^d	5.14 ± 0.09 ^b
Moisture (%)	43.5 ± 1.2 ^a	39.3 ± 1.1 ^{bc}	38.5 ± 1.0 ^c	37.7 ± 1.1 ^d	37.4 ± 1.2 ^d	40.8 ± 1.1 ^b	36.5 ± 1.0 ^e	35.8 ± 1.3 ^e	34.2 ± 1.4 ^f	33.8 ± 1.6 ^f	43.1 ± 1.4 ^a	39.0 ± 1.4 ^{bc}	38.1 ± 1.1 ^c	37.4 ± 1.3 ^d	37.0 ± 1.5 ^d
Fat (%)	40.1 ± 1.1 ^d	41.5 ± 1.3 ^c	41.8 ± 1.5 ^c	42.0 ± 1.5 ^b	42.3 ± 1.8 ^b	41.9 ± 1.5 ^c	42.9 ± 1.7 ^a	43.3 ± 0.9 ^a	43.6 ± 1.1 ^a	43.9 ± 0.7 ^a	40.3 ± 1.0 ^d	41.2 ± 1.0 ^c	41.7 ± 0.9 ^c	42.2 ± 1.1 ^a	42.5 ± 1.2 ^b
Protein (%)	41.0 ± 0.8 ^d	42.1 ± 1.0 ^c	42.3 ± 1.1 ^c	42.6 ± 0.9 ^{bc}	42.9 ± 1.0 ^b	42.1 ± 0.9 ^c	43.5 ± 1.0 ^b	43.9 ± 0.8 ^b	44.3 ± 0.9 ^a	44.7 ± 0.5 ^a	41.2 ± 0.8 ^d	42.3 ± 1.1 ^c	42.5 ± 1.2 ^c	42.9 ± 0.9 ^c	43.1 ± 1.7 ^b
NaCl (%)	1.91 ± 0.1 ^d	1.99 ± 0.1 ^c	2.0 ± 0.2 ^b	2.1 ± 0.1 ^b	2.2 ± 0.2 ^b	2.1 ± 0.2 ^b	2.31 ± 0.1 ^{ab}	2.36 ± 0.2 ^a	2.38 ± 0.2 ^a	2.4 ± 0.2 ^a	1.89 ± 0.1 ^e	1.95 ± 0.1 ^{cd}	1.97 ± 0.1 ^c	1.98 ± 0.1 ^c	2.0 ± 0.1 ^b

^{a-f}Means within a row with different superscript letters are significantly different ($P < 0.05$).¹Mean values ± SD for 3 batches of each type of cheese analyzed in duplicate.**Table 3.** Cell numbers ($\log_{10}$ cfu/g)¹ of presumptive mesophilic lactobacilli and thermophilic streptococci during ripening of Caciocavallo Pugliese cheeses

Item	Control cheese					Cheese with adjunct culture					Cheese with attenuated adjunct culture				
	0 d	15 d	30 d	45 d	60 d	0 d	15 d	30 d	45 d	60 d	0 d	15 d	30 d	45 d	60 d
Mesophilic lactobacilli	3.5 ± 0.3 ^d	4.9 ± 0.3 ^d	6.7 ± 0.4 ^b	7.2 ± 0.4 ^a	7.6 ± 0.3 ^c	6.2 ± 0.3 ^b	8.4 ± 0.4 ^b	8.6 ± 0.3 ^b	8.5 ± 0.3 ^b	8.7 ± 0.3 ^b	4.1 ± 0.3 ^c	6.6 ± 0.4 ^b	8.4 ± 0.3 ^b	8.5 ± 0.4 ^a	8.4 ± 0.3 ^b
Thermophilic streptococci	9.3 ± 0.4 ^b	9.5 ± 0.4 ^a	9.4 ± 0.4 ^a	9.6 ± 0.4 ^a	8.9 ± 0.4 ^c	9.5 ± 0.4 ^a	9.4 ± 0.4 ^a	9.6 ± 0.4 ^a	9.5 ± 0.4 ^c	8.6 ± 0.3 ^c	9.5 ± 0.4 ^a	9.1 ± 0.4 ^b	9.4 ± 0.4 ^a	9.5 ± 0.4 ^a	8.7 ± 0.3 ^c

^{a-d}Means within a row with different superscript letters are significantly different ($P < 0.05$).¹Mean values ± SD for 3 batches of each type of cheese analyzed in duplicate.

Figure 3. Urea-PAGE of pH 4.6-insoluble (A) and pH 4.6-soluble (B) nitrogen fraction of Caciocavallo Pugliese cheeses after 0, 30, and 60 d of ripening. Lane 1 = bovine CN standard; lanes 2, 3, and 4 = control cheese (CC), cheese with adjunct cultures (AC), and cheese with attenuated adjunct cultures (AAC) after 0 d of ripening; lanes 5, 6, and 7 = CC, AC, and AAC after 30 d of ripening; and lanes 8, 9, and 10, CC, AC, and AAC after 60 d of ripening.

made with the addition of AAC. This concentration was approximately 5 times higher than that found in CC ($1,655.2 \pm 25$ mg/kg) and twice that found in the cheese manufactured with AC ($5,875.5 \pm 23$ mg/kg). Previously (Morea et al., 2007), the concentration of total FAA of Caciocavallo Pugliese cheese made with the addition of *L. paracasei* and *L. parabuchneri* strains was found to be 20,954 mg/kg. Several studies reported accelerated or increased liberation of FAA when adjunct or attenuated starters were used in cheese manufacture (Ardö et al., 1989; Madkor et al., 2000; Katsiari et al., 2002; Michaelidou et al., 2003; Upadhyay et al., 2007). Accelerated liberation of most FAA in cheeses promotes increased synthesis of volatile compounds and markedly influences the flavor of cheeses (Fox and Wallace, 1997). It was previously shown (Madkor et al., 2000) that the liberation of FAA by attenuated adjunct cultures is highest after approximately 1 mo of ripening, which corresponds to the time needed to attain a significant extent of cell autolysis. Serine, Glu, Leu, Phe, Lys, and Pro were the FAA found at the highest concentrations, especially in the Caciocavallo Pugliese cheese manufactured with the addition of AAC in the current study. These FAA corresponded to the most common profiles of FAA for Italian cheese varieties (Resmini et al., 1988; Gobetti et al., 1999; Albenzio et al., 2001; Di Cagno et al., 2003).

Concentrations of the same enzyme activities that were previously used to select nonstarter lactobacilli were determined in the water-soluble extracts of the cheeses during ripening (Figure 4, panels A to E). All of these enzymes are important in secondary proteolysis and the catabolism of FAA (Gobetti et al., 1999). A well-defined trend was observed: all enzyme activities were highest in the cheese manufactured with AAC, and the largest significant ($P < 0.05$) differences were found at 30 d of ripening. The enzyme activity levels in AAC cheese were at least twice those found in CC. Further, cystathionine lyase activity was detectable in all cheeses but only at the end of ripening in CC. These results might be related to several of the previous findings. As shown through in vitro characterization, a portion of the AAC population was severely damaged, which promoted the liberation of enzymes in the curd. The remaining viable portion of the AAC population continued to grow over the same period of ripening and may have contributed, even to a small degree, to the activity of the enzymes measured. Moreover, a contribution from autolysed cells of primary starters cannot be excluded. The concentration of total FAA completely agreed with the differences found for enzyme activities. With the exception of proline iminopeptidase activity, the enzyme activities found in the cheese manufactured with AC were significantly ($P < 0.05$) higher than

Table 4. Mean values¹ for the level of free AA (mg/kg) found in Caciocavallo Pugliese cheeses made without adjuncts (control), with adjunct cultures (AC), and with attenuated adjunct cultures (AAC) during ripening

AA (mg/kg)	Control cheese		Cheese with AC		Cheese with AAC	
	0 d	60 d	0 d	60 d	0 d	60 d
Cysteic acid	14.58	11.91	6.70	23.85	10.69	26.25
Asp	11.34	17.89	14.61	56.94	14.05	67.08
Thr	11.67	8.77	11.67	99.18	16.06	191.21
Ser	26.06	77.70	37.41	594.03	60.49	1,090.88
Glu	20.49	26.43	20.02	196.88	25.43	1,366.53
Gly	7.24	22.25	7.82	133.31	11.47	224.77
Ala	27.23	68.01	47.86	322.79	35.40	450.72
Cys	16.24	25.76	10.00	42.05	16.68	85.93
Val	34.07	103.16	42.62	351.15	36.21	517.90
Met	22.65	50.43	26.83	132.98	22.30	208.24
Ile	12.57	29.46	14.56	127.13	19.91	249.01
Leu	48.90	233.90	65.40	1,291.89	51.16	1,686.14
Tyr	49.68	74.87	31.74	185.65	41.60	279.65
Phe	52.60	226.87	38.03	710.42	60.05	944.59
His	46.02	185.60	21.04	140.76	34.91	214.52
Trp	15.89	73.81	7.51	45.75	13.15	50.85
Orn	16.73	162.85	28.49	122.57	19.10	139.40
Lys	97.86	153.38	98.74	522.48	108.94	935.24
Arg	27.49	0.10	15.89	144.27	15.99	402.51
Pro	75.67	102.02	68.07	631.46	72.13	928.70
Total	634.9 ± 25 ^d	1,655.2 ± 66 ^c	678.84 ± 32 ^d	5,875.5 ± 23 ^b	685.7 ± 39 ^d	10,060.1 ± 40 ^a

^{a-d}Means within a row with different superscript letters are significantly different ($P < 0.05$).

¹Mean values ± SD for 3 batches of each type of cheese analyzed in duplicate.

those found in CC. The same was found for peptidase activities in Caciocavallo Pugliese cheese made with other adjunct cultures (Gobbetti et al., 2002).

Sensory Analysis

Acceleration of ripening under standardized conditions is the major goal of using attenuated adjunct cultures (Johnson et al., 1995; Madkor et al., 1999). It is expected that sensory properties of the cheese when ripening is accelerated would not differ from those achieved under conventional maturation. Based on the above results, the sensory properties of the Caciocavallo Pugliese manufactured with AAC after 45 d of ripening were compared, by triangle test (ISO, 2004), with those of CC after 60 d of ripening. The 2 cheeses did not differ significantly. Only 18 of 42 assessors recognized the 2 cheeses as different, a value lower than the expected number (20) of correct responses ($\alpha = 0.05$, $\beta = 0.05$, and Pd 40%) required to determine that a significant difference was noticeable by panelists. The opposite was found when CC (60 d of ripening) was compared with AC (45 d) and AC (45 d) was compared with AAC (45 d). Based on the same parameters of sensitivity, respectively, 26 and 25 assessors recognized these pairs as being different cheeses. These values were higher than the expected number (20) of correct responses. Based on the results of this study, the use of AAC was suitable

for accelerating the ripening of Caciocavallo Pugliese cheese without adverse effects on sensory properties.

CONCLUSIONS

The NSLAB represent an appreciable source of diversity and enzyme activities to be used in cheesemaking. Nevertheless, their effect on cheese quality is variable depending on the strain and procedure used. Although still contentious, attenuation is one of the most promising procedures to use selected NSLAB under controlled and consistent conditions. This study showed that sonication is useful tool to achieve attenuation. The effect of sonication on the cell membrane integrity of nonstarter lactobacilli was characterized in vitro and confirmed during cheesemaking. Attenuated adjunct cultures were suitable for making ripened *pasta filata* cheeses such as Caciocavallo Pugliese. As shown through microbiological, biochemical, and sensory analyses, the accelerated ripening of Caciocavallo Pugliese cheese was achieved without modification of the main features of the traditional cheese made without adjuncts.

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Figure 4. Enzymatic activities (arbitrary units/kg) of water-soluble extracts of Caciocavallo Pugliese cheeses made without adjunct cultures (control; black bars), with adjunct cultures (light gray bars), and with attenuated adjunct cultures (dark gray bars) after 0, 15, 30, 45, and 60 d of ripening. Panels show activities of aminopeptidase type N (A), iminopeptidase (B), endopeptidase type O (C), glutamate dehydrogenase (D), and cystathionine lyase (E). Values represent the average of 3 triplicates ($\pm$ SD) from 3 cheese-making experiments.

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